A longitudinal study of *Giardia duodenalis* genotypes in dairy cows from birth to 2 years of age

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A B S T R A C T

Fecal specimens were collected from 30 calves from birth to 24 months of age at a dairy farm in Maryland to determine the prevalence of *Giardia duodenalis* genotypes in cattle of different ages. Fecal samples were subjected to density gradient centrifugation to remove debris and concentrate cysts. Specimens were analyzed by immunofluorescence microscopy and polymerase chain reaction (PCR). All PCR positive specimens were sequenced using the SSU-rRNA gene of *Giardia*. All 30 calves shed *G. duodenalis* cysts at some time during the study. Of 990 specimens, 312 were positive for *G. duodenalis* (31.5%). The highest prevalence of infection occurred at weeks 4 and 5 of age with 25 out of 30 calves shedding cysts at those sampling times. Overall, pre-weaned calves (<8 weeks of age) exhibited the highest prevalence (60.8%), followed by post-weaned calves (3–12 months of age) (32.1%) and heifers (12–24 months of age) (11.4%). Sequence analysis of the 312 PCR-positive samples revealed the presence of both Assemblages A and E, *G. duodenalis*, with cumulative prevalences of 70% and 100%, respectively. Assemblage A was not detected in pre-weaned calves, but was detected in 6.9% and 4.7% of post-weaned calves and heifers, respectively. These data indicate not only that calves are infected with both Assemblages A and E simultaneously, but also that infections with zoonotic Assemblage A, *G. duodenalis* are more common than previously reported. Thus, calves appear to be a more significant reservoir of human infectious *G. duodenalis* than previous data have suggested.

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1. Introduction

Giardia duodenalis (syn. *Giardia lamblia, Giardia intestinalis*) is a common intestinal parasite of mammals, including humans. Molecular characterization of isolates has revealed seven major genotypes (Assemblages) of *G. duodenalis* that appear to have different host ranges (Monis et al., 1999, 2003; Thompson et al., 2000; Thompson and Monis, 2004). Three of these Assemblages, A, B, and E, have been detected in cattle; Assemblages A and B also infect humans (Monis et al., 2003; Thompson and Monis, 2004).

Thus there is concern that cattle could represent a reservoir of *G. duodenalis* with the potential to cause disease in humans either through direct contact or by contamination of food and water supplies. Although an increasing number of studies have used molecular techniques to determine the prevalence of *G. duodenalis* genotypes in cattle, there is still relatively little information available regarding what changes, if any, occur in these genotypes over time in a particular group of animals.

Point prevalence studies demonstrate considerable variation in the levels of infection in cattle, although longitudinal studies frequently report cumulative prevalences of 100% (Xiao and Herd, 1994; Olson et al., 1997a,b; Ruest et al., 1998; O'Handley et al., 1999; Ralston et al., 2003; Castro-Hermida et al., 2006; Gow and Waldner,

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2006; Hamnes et al., 2006). Such prevalence data based on microscopic analysis is easily obtained; however, molecular prevalence data on *G. duodenalis* genotypes in cattle is not as readily available. Surveys of dairy cattle in Canada, Australia, and the Netherlands have reported predominately Assemblage E, with lower levels of Assemblage A (O'Handley et al., 2000; Huetink et al., 2001; Appelbee et al., 2003) Assemblage B has been reported in cattle in Italy, Canada, New Zealand, and Portugal (Lalle et al., 2005; Coklin et al., 2007; Mendonça et al., 2007; Winkworth et al., 2008).

Four sequential multi-state prevalence studies for *G. duodenalis* in the eastern United States sampling a total of almost 2000 animals from birth to adulthood, reported that pre-weaned calves (<2 months of age), post-weaned calves (2–12 months of age), heifers (13–24 months of age), and adult cows were infected with both Assemblages E and A, *G. duodenalis* (Trout et al., 2004, 2005, 2006, 2007). Although there was significant farm to farm variation, overall, Assemblage E was found in 28% of pre-weaned calves, 45% of post-weaned calves, 33% of heifers, and 25% of adult cows, whereas Assemblage A, was detected in 6% of pre-weaned calves, 7% of post-weaned calves, 3% of heifers, and 2% of adult cows. Similar data on the prevalence of genotypes present in different age groups sampled longitudinally are not available. A recent longitudinal study conducted on adult dairy cows revealed that on average 49% of the cows was positive for *G. duodenalis* (Uehlinger et al., 2006). Of 14 isolates that were sequenced, 6 (43%) were Assemblage A and 8 (57%) were Assemblage E. Thus the current study was undertaken to follow the same group of 30 dairy cows from birth to 2 years of age and determine changes in the prevalence of *G. duodenalis* genotypes over time, as well and the cumulative prevalence of the genotypes.

2. Materials and methods

2.1. Animals and collection of specimens

This study was conducted under an animal use protocol approved by the Beltsville Area Animal Care and Use Committee. Thirty newborn calves from a dairy farm in Maryland were randomly selected and included in the study group. All the calves were purebred Holstein females born from different cows over a period of 5 months (November 2004–March 2005). Each calf was removed from its dam within 1 h after birth and initially housed in an individual hutch approximately 100 m from the cows. The *Giardia* history of the dams is unknown. From 3 months of age through 24 months of age, the calves were housed in groups in large pens, partially covered with roofs for shelter. Calves were considered pre-weaned from birth until 8 weeks of age, post-weaned from 3–12 months of age, and heifers from 13–24 months of age. Fecal specimens were collected weekly from calves up to 8 weeks of age, bi-weekly from calves 3–5 months of age, and monthly from animals 6–24 months of age. Feces were collected directly from the rectum of each animal into a plastic cup. The cups were sealed, labeled, and immediately placed onto ice or cold packs in an insulated

container. Specimens were transported to the laboratory processed no later than 3 days after collection.

2.2. Concentration of cysts from feces

Feces were processed as described by Trout et al. (2004). Briefly, 15 g of feces from each sample were mixed with 35 ml of distilled water (dH_2O), and the suspension was passed through a wire mesh sieve with a 45 μm pore size and transferred to a 50 ml conical tube. The volume was adjusted to 50 ml with dH_2O and centrifuged at $1800 \times g$ for 15 min. The resulting pellet was suspended in 25 ml dH_2O; 25 ml CsCl (1.4 g/ml) was added, and the tubes were thoroughly mixed by vortexing. The samples were centrifuged at $300 \times g$ for 20 min. The topmost 4 ml of supernatant was aspirated from each tube, washed twice with dH_2O and the final pellet was suspended in 500 μl of dH_2O. The resulting samples were examined by both microscopy and molecular methods as described below.

2.3. Immunofluorescence microscopy (IFA)

A 100 μl aliquot of the processed feces was transferred to a microcentrifuge tube and washed once with dH_2O. The pellet was suspended in 50 μl of premixed Mer*IF*luor[TM] reagents (Meridian Biosciences, Cincinnati, OH) and 2 μl of this suspension was placed onto one well (11 mm diameter) of a 3-well, Teflon-coated, glass microscope slide (Erie Scientific, Portsmouth, NH). The slide was covered with a 24 mm × 50 mm coverslip and the entire well area was examined at 400× magnification using a Zeiss Axioskop equipped with epifluorescence and an FITC-Texas Red[TM] dual wavelength filter.

2.4. DNA extraction

Total DNA was extracted from each CsCl-cleaned fecal sample using a DNeasy Tissue Kit (Qiagen, Valencia, CA) with a slightly modified protocol. The protocol, described below, utilized proprietary reagents provided by the manufacturer. A total of 50 μl of processed feces was suspended in 180 μl of ATL buffer and thoroughly mixed by vortexing. Twenty microliters of proteinase K (20 mg/ml) was added, and the suspension was again thoroughly mixed. Following an overnight incubation of the mixture at 55 °C, 200 μl of AL buffer was added. The remaining protocol followed manufacturer's instructions with one exception. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μl of AE buffer.

2.5. PCR and DNA sequence analysis

A 292 bp fragment of the SSU-rRNA gene was amplified using PCR as previously described (Hopkins et al., 1997); products were analyzed on 1% agarose gel with ethidium bromide staining. All positive PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT[TM]) (USB Corporation, Cleveland, OH) and sequenced in both directions using the same PCR primers in 10 μl reactions, Big Dye[TM] chemistries, and an ABI 3100 sequencer analyzer (Applied Biosystems, Foster City, CA).
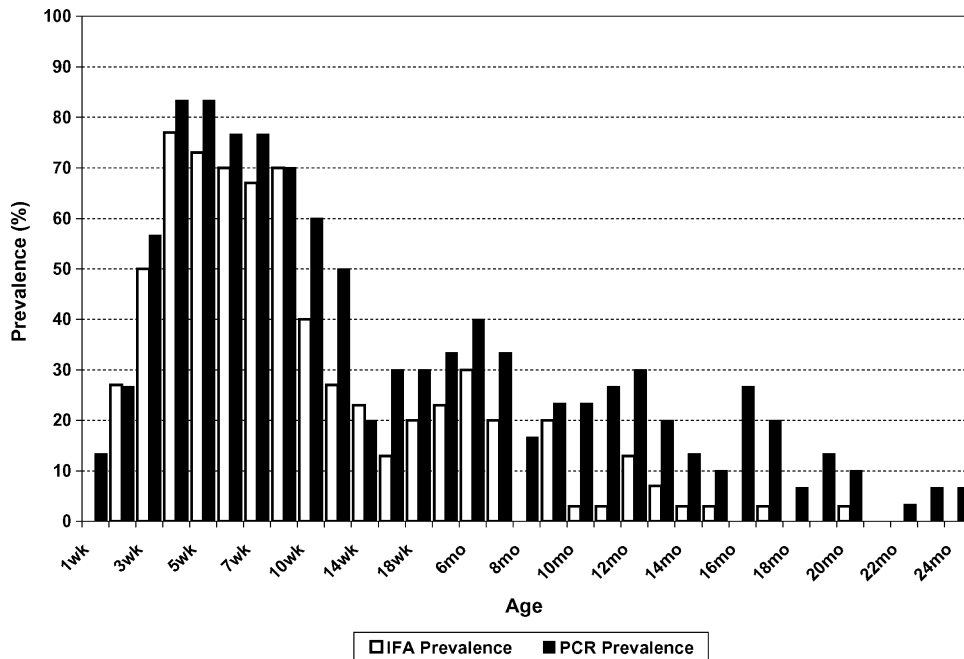
Fig. 1. Prevalence of *Giardia* spp. in a group of 30 dairy cattle sampled from 1 week through 24 months of age, as determined by immunofluorescence microscopy and PCR.

Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI).

3. Results

3.1. Prevalence of Giardia

A total of 990 samples were collected over the course of the study. PCR provided more sensitive detection: a total of 209 (21.1%) *Giardia* positive samples were detected by IFA, and 312 (31.5%) were detected by PCR. The percentages of animals positive for *Giardia* by IFA and PCR determined at weekly, bi-weekly, and monthly intervals are shown in Fig. 1. The number of *Giardia* infected animals as determined by PCR rose rapidly from birth, peaking at 25 of 30 calves (83.3%) at both 4 and 5 weeks of age. Three progressively smaller peaks in prevalence were observed at 6 months (40%), 12 months (33.3%), and 16 months (23.3%). By age group, *Giardia* infections were detected in 60.8% of pre-weaned calves, 32.1% of post-weaned calves, and 11.4% of heifers. Over the course of the study all calves became infected.

3.2. Sequence analysis of the SSU-rRNA gene

Sequence analysis of the 312 PCR-positive samples revealed the presence of Assemblage A in 44 samples (4.4%) and Assemblage E in 266 samples (26.9%). Two samples (0.2%) were clearly mixed infections of both A and E. The percentages of animals positive for Assemblages A and E *Giardia* determined at weekly, bi-weekly, and monthly intervals are shown in Fig. 2. Only Assemblage

E was found in calves less than 18 weeks of age and contributed to the initial peak in *Giardia* prevalence; Assemblage A was first detected at 18 weeks of age. The highest prevalence of Assemblage E (83.3%) was observed at 4 and 5 weeks of age, whereas the highest levels of Assemblage A (16.7%) was observed at 18 weeks and 6 months of age. Nine animals shed exclusively Assemblage E. The other 21 animals initially shed Assemblage E, followed by Assemblage A, or Assemblage A alternating with E at different sampling times. Two mixed infections (not included in figures) were detected in the same animal at both 17 and 18 months of age. The prevalence of Assemblages A and E, by age group is shown in Fig. 3. For Assemblage E, 60.8%, 25.1%, and 6.1% of pre-weaned calves, post-weaned calves, and heifers were infected, respectively; for Assemblage A, 0%, 6.9%, and 4.7% of pre-weaned calves, post-weaned calves, and heifers were infected, respectively. The cumulative prevalence of Assemblage E reached 100% by 7 weeks of age, and the cumulative prevalence of Assemblage A reached a high of 70% by 15 months of age.

4. Discussion

Of the 990 fecal specimens from 30 calves collected from the same 30 dairy calves from birth to 2 years of age, PCR detected approximately 10% more infections than IFA. The discrepancy between IFA and PCR increased as the age of the animals increased, perhaps due to lower numbers of cysts/g in older animals.

The highest prevalence of 83.3% was found at 4 and 5 weeks of age. The cumulative prevalence reached 100% by 7 weeks of age. A 100% infection rate has been reported in
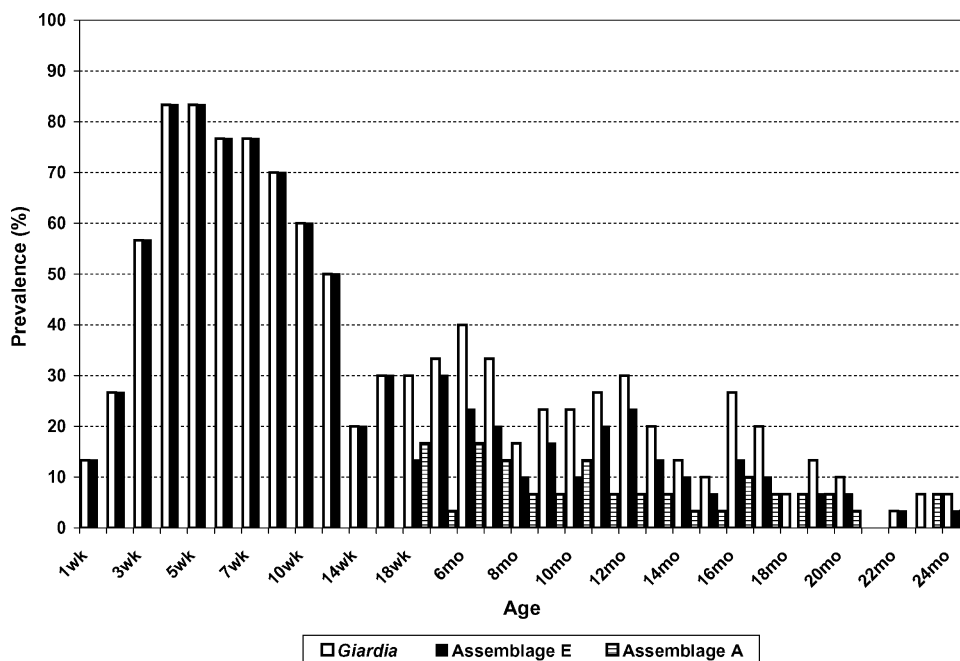
Fig. 2. Prevalence of total *Giardia* as well as Assemblages A and E in a group of 30 dairy cattle sampled from 1 week through 24 months of age.

previous longitudinal studies (Xiao and Herd, 1994; O'Handley et al., 1999). The variation seen at different ages in Fig. 1 provides, in part, an explanation for the differences in point prevalence studies reported in numerous published studies (Xiao, 1994; Olson et al., 1997a,b; O'Handley et al., 2000; Trout et al., 2004, 2005, 2006, 2007; Castro-Hermida et al., 2006; Gow and Waldner, 2006; Hamnes et al., 2006). The great variation in prevalence at different ages coupled with the intermittent nature of cyst excretion (Buret et al., 1990)

demonstrate the likelihood that point prevalence studies underestimate the actual number of infected animals. Additionally, no technique is likely to be sufficiently sensitive to detect very low levels of cyst production, thus invariably false negatives occur, further underestimating the actual prevalence.

Giardia infections were detected from 1 week through 24 months of age. The prevalence peaked at 4–8 weeks of age. Infections found after 7 weeks of age when all calves had become infected, were either due to the inability to
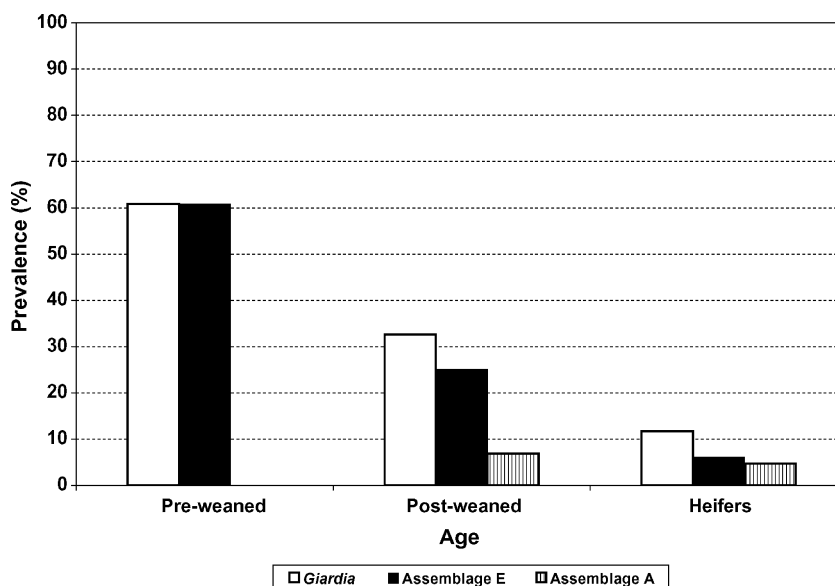

Fig. 3. Prevalence of total *Giardia* and Assemblages A and E, in a group of 30 dairy cattle sampled from 1 week through 24 months of age, compared by age group: pre-weaned, 1–8 weeks of age; post-weaned, 3–12 months of age; heifers, 13–24 months of age.

clear initial infection or to reinfection. The prevalence of infections, however, did decrease over time, from a high of 60.8% in pre-weaned calves, to 32.1% in post-weaned calves, to 11.4% in heifers. Previous research in our laboratory, wherein pre-weaned calves, post-weaned calves, and heifers on 14 farms in the eastern United States were sampled in consecutive years, indicated that *Giardia* infections were most prevalent in post-weaned calves (Trout et al., 2004, 2005, 2006). A direct comparison between the present longitudinal study and earlier point prevalence studies is not possible because of the limitation of sample numbers and differences in animal age, housing conditions and many other environmental and management factors.

Despite the fact that calves were removed from their dams within 1 h after birth and housed individually with no contact between animals for the first 3 months of life, all developed *Giardia* infections by 7 weeks of age. The sources of cysts initiating infections in these calves are unclear. Cross-contamination between the animals before samples were collected cannot be ruled out due to environmental contamination or directly from one calf to another during the course of the study. The prepatent period for *Giardia* in experimental infections is generally 7–8 days (Taminelli et al., 1989). Since some calves became infected after this time period, it is unlikely that initial contact with the cow was the source of the infections. For *Cryptosporidium*, various housing surfaces have been reported to be sources of infectious oocysts (Atwill et al., 1998), and mechanical vectors such as birds and flies have the potential to transport both *Cryptosporidium* oocysts and *Giardia* cysts (Szostakowska et al., 2004; Conn et al., 2007).

Both Assemblages A and E of *G. duodenalis* were detected. Only Assemblage E was found during the initial peak in prevalence that extended from 4 to 8 weeks of age; Assemblage A was first detected at 18 weeks of age and exhibited its highest prevalence at this age and again when animals were 6 months of age (16.7%). When examined by age group, Assemblage E was detected in 60.8% of pre-weaned calves, 25.1% of post-weaned calves and 6.1% of heifers, whereas Assemblage A was detected in 0%, 6.9%, and 4.7%, of these same age groups, respectively. These findings differ from pooled point prevalence studies on 14 farms wherein the prevalence of Assemblage E was highest in post-weaned calves and Assemblage A was detected in pre-weaned calves (Trout et al., 2004, 2005, 2006).

Additional studies have found that calves harbor primarily Assemblage E with generally low levels of Assemblage A (O'Handley et al., 2000; Huetink et al., 2001; Appelbee et al., 2003). However, when the cumulative prevalence of these two assemblages in the present study was calculated Assemblage E reached 100% by 7 weeks of age, as might be expected based on previous point prevalence studies. The cumulative prevalence of Assemblage A reached 70% by 15 months of age. This finding was somewhat unexpected considering the low levels this assemblage previously reported in cattle, however a recent longitudinal study of adult cows reported 43% of the positive isolates were Assemblage A and 57% were Assemblage E (Uehlinger et al., 2006). The higher levels of Assemblage A detected in longitudinal studies, raise

several important points. Infections with Assemblages E and A can occur simultaneously or consecutively; this is further supported by detection of two mixed infections in the current study. The presence of mixed infections is confirmed by at least one other report, wherein an assemblage-specific PCR detected both Assemblages A and E in the same animals (Geurden et al., 2007). Such a protocol can detect mixed infections even when one assemblage is present at much higher levels; the genotyping protocol used in the current study, as well as numerous others, will generally detect mixed infections only if the assemblages are present in roughly equal numbers, otherwise, only the predominate genotype is detected. Thus, it appears that Assemblage A is being underreported because Assemblage E is present at higher levels. Indeed Geurden et al. (2007) found higher levels of Assemblage A than have typically been reported, and a recent study found only Assemblages A and B in dairy calves in New Zealand (Winkworth et al., 2008). Data on individual animals in the current study revealed that only nine animals shed exclusively Assemblage E. The remaining 21 animals shed both genotypes; Assemblage E was detected first, then Assemblage A and frequently the two genotypes were detected alternately at subsequent sampling times. It appears that the initial infection with Assemblage E does not provide any form of protection against infections with Assemblage A; it is also possible that Assemblage A infections are present in the first weeks after birth, but are simply obscured by high amounts of Assemblage E.

The current study provides a comprehensive examination of the prevalence of *G. duodenalis* genotypes in a group of 30 dairy cows from birth through 2 years of age. The data confirm not only that co-infections of Assemblages A and E occur, but also suggest that the prevalence of Assemblage A in dairy cattle has been considerably underestimated by point prevalence studies. The highest prevalence of Assemblage A at any sampling point in the current study was 16.7%, however, cumulative data demonstrates that by 15 months of age, 70% of the animals became infected with Assemblage A. Additional longitudinal studies are necessary to determine if the data in the present study is representative of dairy farms in general, or specific only to the study farm. If Assemblage A is routinely present at a high prevalence, then the role of dairy cattle as a reservoir for *Giardia* capable of infecting humans should be considered.

Acknowledgments

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